Identification of Aminotadalafil and its Stereoisomers Contained in Health Foods Using Chiral Liquid Chromatography-Mass Spectrometry

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Using chiral liquid chromatography-mass spectrometry (LC/MS), a simple and rapid identification test was developed for aminotadalafil [(6*R*,12a*R*)-2-amino-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydropyrazino[1′,2′ : 1,6]pyrido[3,4-*b*]indole-1,4-dione; *RR*-ATDF] and its stereoisomers contained in health foods, *e.g.* herbal products. A sample solution was prepared using methanol extraction. Analysis was performed on a chiral column with the mixture of 0.1% formic acid/acetonitrile (7 : 3) as mobile phase at 30°C. Each resolution value of four stereoisomers of ATDF was greater than 1.3. A mass spectrometer was used as a detector to enhance specificity by excluding the effects of general components derived from the sample. The four individual stereoisomers of ATDF in the health foods were identified based on their respective retention times. Results showed that structural conversions of *RR*-ATDF into (6*R*,12a*S*)-2-amino-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydropyrazino[1′,2′ : 1,6]pyrido[3,4-*b*]indole-1,4-dione (*RS*-ATDF) and (6*S*,12a*S*)-2-amino-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydropyrazino[1′,2′ : 1,6]pyrido[3,4-*b*]indole-1,4-dione (*SS*-ATDF) into (6*S*,12a*R*)-2-amino-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydropyrazino[1′,2′ : 1,6]pyrido[3,4-*b*]indole-1,4-dione (*SR*-ATDF) occurred under strongly basic conditions, which indicates that such conditions must be avoided during sample preparation. Using this method, *RR*-ATDF and *SR*-ATDF were detected in a health food; this is the first report describing that ATDF diastereomers are present in health foods.

Key words —— aminotadalafil, stereoisomer, chiral column, liquid chromatography-mass spectrometry, identification test, health food

INTRODUCTION

According to a report of the Ministry of Health, Labour and Welfare, the addition of phosphodiesterase type-5 inhibitor, the principal component of erectile dysfunction drugs such as sildenafil and tadalafil, into so-called health foods that are marketed and advertised as roborants happens frequently. Structural analogs that have a partly modified structure of pharmaceuticals have been detected in some cases.[1–3] Although such analogs are considered to possess the same activities as their original compounds, little is known about their actual physiological activities. Furthermore, very little information is available about stereoisomers in many *nonpharmaceutical* organic compounds that have asymmetric carbons. These chemical compounds are not subjected to an official review process as are pharmaceuticals. Therefore, they present risks of severe health damage because of their unpredictable activities. For health risk management, it is important and urgently necessary to collect information about such stereoisomers and to develop identification methods for them.

Aminotadalafil [(6*R*,12a*R*)-2-amino-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydropyrazino[1′,2′ : 1,6]pyrido[3,4-*b*]indole-1,4-dione; *RR*-ATDF shown in Fig. 1] is a structural analog of tadalafil. It has two asymmetric carbons; theoretically, two pairs of enantiomers exist. In fact, ATDF is detected repeatedly in the health foods marketed

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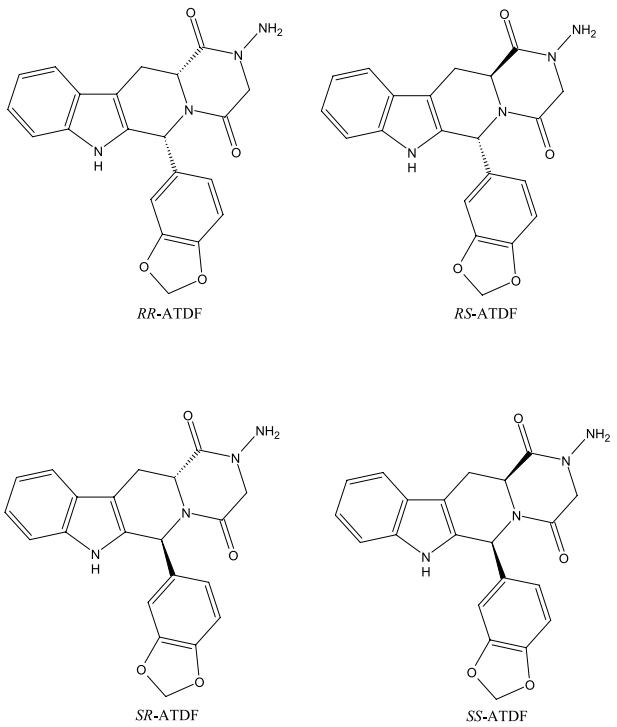

Fig. 1. Chemical Structures of Aminotadalafil and Its Stereoisomers

in and outside of Japan. For that reason, it is an important object for administrative measures. During analysis of a health food containing ATDF, we detected a compound which has a similar UV and mass spectrum to those of ATDF. The compound is adjacent to the peak of ATDF on reverse-phase high-performance liquid chromatography (HPLC) and seems to be a diastereomer of ATDF. Therefore, to establish identification analysis for the diastereomers and enantiomers of ATDF, we synthesized individual stereoisomers and investigated the possibility of their separation using chiral HPLC;[4] in the present study, we identified a method of performing simultaneous analysis of the four stereoisomers. Furthermore, we found that the structural conversions of *RR*-ATDF into (6*R*,12a*S*)-2-amino-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydropyrazino[1′,2′ : 1,6]pyrido[3,4-*b*]indole-1,4-dione (*RS*-ATDF) and (6*S*,12a*S*)-2-amino-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydropyrazino[1′,2′ : 1,6]pyrido[3,4-*b*]indole-1,4-dione (*SS*-ATDF) into (6*S*,12a*R*)-2-amino-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydropyrazino[1′,2′ : 1,6]pyrido[3,4-*b*]indole-1,4-dione (*SR*-ATDF) occur under a strongly basic condition.

MATERIALS AND METHODS

Sample and Reagents —— Contents of an encapsulated health food manufactured in the U.S.A. and imported to Shizuoka prefecture were subjected to analyses.

D-Tryptophan methyl ester hydrochloride, L-tryptophan methyl ester hydrochloride and hydrazine monohydrate were purchased from Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.). Trifluoroacetic acid, piperonal and chloroacetyl chloride were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Liquid chromatography-mass spectrometry (LC/MS) grade acetonitrile was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals were of analytical grade.

Sample Solution Preparation —— For chiral HPLC analysis, ATDF was extracted from 50 mg test sample with 100 ml of methanol using ultrasonication; then the extract solution was filtrated using a 0.45 μm membrane filter.

Synthesis of ATDF and Its Stereoisomers —— First, *RR*-ATDF and its stereoisomers (Fig. 1) were synthesized from D-tryptophan and L-tryptophan methyl esters as starting materials using the method described by Daugan *et al.*[5] and a relevant patented method (WO 02/00656). For purification, these compounds were applied to an aminopropylated silica gel plate (NH TLC plate; Fuji Silysia Chemical Ltd., Kasugai, Japan) and were developed using ethyl acetate. The spot was scraped and eluted using ethyl acetate. Then the elute was evaporated and dried in vacuum. For ^{1}H-NMR, ATDF stereoisomers (13 mg/ml) were prepared in dimethyl sulfoxide (DMSO) and measured at room temperature using a spectrometer (JNM-AL400HG; JEOL, Tokyo, Japan). For optical rotation determination, ATDF stereoisomers (7 mg/ml) were prepared in DMSO and filled into a 100-mm-long polarimeter tube. The angular rotation was measured using a polarimeter (SEPA-300; Horiba Ltd., Kyoto, Japan) with the D line of sodium as the light source at 25°C. The specific rotation was calculated.

RR-ATDF: ^{1}H-NMR (400 MHz, DMSO-d_6) δ 11.02 (1 H, s), 7.55 (1 H, d, J = 7.6 Hz), 7.29 (1 H, d, J = 8.0 Hz), 7.07–6.98 (2 H, m), 6.87 (1 H, s), 6.82–6.76 (2 H, m), 6.09 (1 H, s), 5.93 (2 H, d, J = 1.6 Hz), 5.12 (2 H, s), 4.44 (1 H, dd, J = 11.6, 3.6 Hz), 4.26 (1 H, d, J = 17.2 Hz), 3.96 (1 H, d, J = 17.2 Hz), 3.57 (1 H, dd, J = 15.6, 4.0 Hz), 3.00 (1 H, dd, J = 15.6, 11.6 Hz). $[\alpha]_D^{25}$+78 (*c* 1.0,

DMSO).

SR-ATDF: ^{1}H-NMR (400 MHz, DMSO-d_6) δ 11.08 (1 H, s), 7.51 (1 H, d, J = 8.0 Hz), 7.32 (1 H, d, J = 8.0 Hz), 7.11 (1 H, t, J = 7.2 Hz), 7.03 (1 H, t, J = 7.2 Hz), 6.89–6.77 (3 H, m), 6.62 (1 H, d, J = 8.0 Hz), 6.01 (2 H, d, J = 5.6 Hz), 5.04 (2 H, s), 4.34 (1 H, d, J = 16.8 Hz), 4.14 (1 H, dd, J = 11.6, 4.0 Hz), 4.07 (1 H, d, J = 17.6 Hz), 3.29 (1 H, dd, J = 15.2, 4.0 Hz), 2.95 (1 H, dd, J = 14.4, 12.0 Hz). $[\alpha]_D^{25}$+231 (c 1.0, DMSO).

RS-ATDF: ^{1}H-NMR (400 MHz, DMSO-d_6) δ 11.08 (1 H, s), 7.51 (1 H, d, J = 7.6 Hz), 7.32 (1 H, d, J = 8.0 Hz), 7.11 (1 H, t, J = 7.2 Hz), 7.03 (1 H, t, J = 7.2 Hz), 6.89–6.76 (3 H, m), 6.62 (1 H, d, J = 8.0 Hz), 6.01 (2 H, d, J = 5.6 Hz), 5.04 (2 H, s), 4.34 (1 H, d, J = 17.2 Hz), 4.14 (1 H, dd, J = 12.0, 4.0 Hz), 4.07 (1 H, d, J = 17.2 Hz), 3.29 (1 H, dd, J = 15.2, 4.0 Hz), 2.95 (1 H, dd, J = 15.2, 12.0 Hz). $[\alpha]_D^{25}$−234 (c 1.0, DMSO).

SS-ATDF: ^{1}H-NMR (400 MHz, DMSO-d_6) δ 11.03 (1 H, s), 7.55 (1 H, d, J = 7.6 Hz), 7.29 (1 H, d, J = 8.0 Hz), 7.08–6.98 (2 H, m), 6.87 (1 H, s), 6.82–6.77 (2 H, m), 6.09 (1 H, s), 5.93 (2 H, d, J = 2.4 Hz), 5.12 (2 H, s), 4.45 (1 H, dd, J = 11.6, 4.0 Hz), 4.27 (1 H, d, J = 16.8 Hz), 3.96 (1 H, d, J = 16.8 Hz), 3.57 (1 H, dd, J = 16.0, 4.4 Hz), 3.00 (1 H, dd, J = 15.6, 11.6 Hz). $[\alpha]_D^{25}$−76 (c 1.0, DMSO).

Chiral HPLC Analysis —— The HPLC system (Waters Corp., Milford, MA, U.S.A.) was equipped with a separation module (Alliance 2695; Waters), a photodiode array detector (PDA, model 2996; Waters) and a mass detector (quattro micro API; Waters). Chiral HPLC analysis was performed using a chiral column (5 μm, 150 × 4.6 mm, Chiralcel OD-RH; Daicel Chemical Industries Ltd., Osaka, Japan) at 30°C. The isocratic mobile phase was a mixture of 0.1% formic acid/acetonitrile (7 : 3). The flow rate was 0.7 ml/min, split into 0.2 ml/min for mass analysis. The PDA wavelength was set as 200–340 nm. Mass analysis was performed with selected ion recording (SIR) mode; the monitor ion was m/z 391.

Stability of ATDF Stereoisomers —— For stability testing of ATDF stereoisomers in relation to the extraction process, 40 μg/ml of each compound solution was prepared using extraction solvent and stored at room temperature. A small amount of solution was applied to chiral HPLC at certain times. For stability testing of ATDF stereoisomers in the liquid-liquid extraction process, 40 μg of each stereoisomer was mixed with 2 ml of 28% aqueous ammonia for 5 min and extracted four times using 4 ml of ethyl acetate. The ethyl acetate layers were collected and evaporated. Then the residue was resolved with 1 ml of methanol and applied to chiral HPLC. The stability was evaluated with the area ratio of each stereoisomer measured using a PDA detector at 285 nm.

RESULTS AND DISCUSSION

Synthesis of ATDF and Its Stereoisomers

Both *RR*-ATDF and *SR*-ATDF were synthesized from D-tryptophan methyl ester; *RS*-ATDF and *SS*-ATDF were synthesized from L-tryptophan methyl ester. Based on results of NMR analysis and measurements of the optical rotation of these stereoisomers, *RR*-ATDF proved to be identical to the compound described in the patent (WO 02/00656). As Fig. 1 shows, we identified individual structures of the other compounds from the NMR spectra and optical rotation measurements.

Separation of ATDF Stereoisomers Using a Chiral HPLC System

In general, diastereomers are separable by TLC or reverse-phase HPLC, whereas enantiomers are not. For that reason, we examined whether the four stereoisomers are separable using chiral HPLC. Using the Chiralcel OD-RH column (4.6 × 150 mm, 5 μm), mixture of 0.1% formic acid/acetonitrile (7 : 3) as a mobile phase, and a flow rate of 0.7 ml/min at 30°C, the four stereoisomers of ATDF were eluted from the chiral HPLC in a sequence of *RS*-ATDF, *SS*-ATDF, *RR*-ATDF, and *SR*-ATDF with the resolution value being greater than 1.3 (Fig. 2). In addition, when a mass spectrometer was used

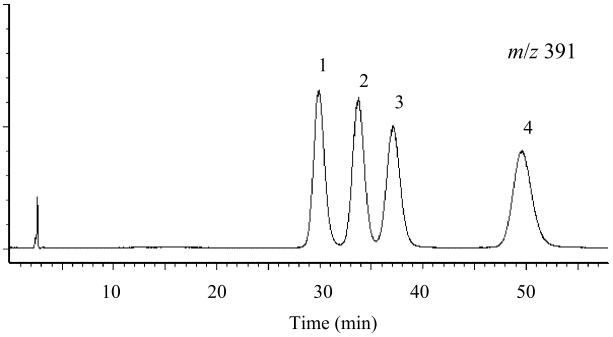

Fig. 2. Separation of Aminotadarafil Stereoisomers Using Chiral HPLC

Peaks 1, *RS*-ATDF; 2, *SS*-ATDF; 3, *RR*-ATDF; and 4, *SR*-ATDF.

as the detector to enhance specificity by excluding the effects of general components derived from the sample, we were able to identify the four individual stereoisomers of ATDF in the health foods based on their respective retention times.

Stability of ATDF Stereoisomers

Optically active compounds might racemize. Consequently, the composition of ATDF and its stereoisomers might change during the analytical process. Therefore, we studied the stability of the stereoisomers during their extraction from the sample. First, according to the official method for determination of ATDF (Director Notice, 2005, Compliance and Narcotics Division, Pharmaceutical and Medical Safety Bureau, Ministry of Health, Labour and Welfare, Japan), we evaluated the stereoisomers' stability in the liquid-liquid extraction process in ethyl acetate following their mixture with 28% aqueous ammonia. Results showed that approximately 5% of *RR*-ATDF and *SS*-ATDF were converted, respectively, into *RS*-ATDF and *SR*-ATDF, although such a composition change was not observed with *SR*-ATDF or *RS*-ATDF (Table 1), probably because of hydrogen exchange at the C12a position under the strongly basic condition. For that reason, the composition change of the stereoisomers was examined in 28% aqueous ammonia/methanol mixed solvent (4 : 1). Results showed that approximately 5%, 50%, and 90% of both the conversions of *RR*-ATDF into *RS*-ATDF and of *SS*-ATDF into *SR*-ATDF occurred in a one-way manner, respectively, during 5 min, 1 hr, and 4 hr after starting the incubation (Table 1). Next, the stability of the stereoisomers in methanol or 1% formic acid/acetonitrile mixed solvents (1 : 4) was studied to evaluate the validity of extraction processes using these solvents. The composition change of the stereoisomers was not observed for 20 hr in these two solvents at room temperature, suggesting that the stereoisomers can be analyzed stably using these extraction solvents (Table 1). These results indicate that a strongly basic condition should be avoided during sample preparation for identification test.

In processes such as purification, where a strongly basic solvent was used for extraction, we were able to avoid such conversion by adding water and an organic solvent to the test sample, with subsequent addition of 28% aqueous ammonia (data not shown).

Health Food Analysis

A health food sample containing ATDF was extracted using methanol. The solvent was analyzed using chiral HPLC. Results indicate that *RR*-ATDF and its diastereomer, *SR*-ATDF, were present and that their peak area ratio is about 4 : 1 (approximately 40 mg and 10 mg per capsule, respectively). Considering the fact that both compounds are synthesized from an identical optically active starting material (D-tryptophan methyl ester) and because no mutual structural conversion of these compounds was found under the current conditions, these results suggest that *SR*-ATDF in the sample was the residue of a by-product that remained after insufficient purification.

For most administrative measures, the identification of unapproved drugs' individual enantiomers is not considered necessary. However, because physiological activities are expected to vary among stereoisomers, it has recently become important to control such stereoisomers to ensure pharmaceuticals' safety. For example, tadalafil, an erectile dysfunction drug which has a structure resembling ATDF, was developed as an optically homogeneous pharmaceutical because the inhibitory effects on phosphodiesterase type-5, *i.e.*, the main effect of tadalafil, vary greatly among its four stereoiso-

Table 1. Conformation Change of Aminotadalafil and Its Stereoisomers in Various Solvents

Conditions	Composition of Aminotadalafil Stereoisomers (%)							
	RR				*SS*			
	RR	*SR*	*RS*	*SS*	*RR*	*SR*	*RS*	*SS*
Mixed with 28% ammonia and extracted with ethyl acetate	95	0	5	0	0	4	0	96
Resoluble in 28% ammonia/methanol (4 : 1) for 5 min	94	0	6	0	0	6	0	94
Resoluble in 28% ammonia/methanol (4 : 1) for 1 hr	49	0	51	0	0	51	0	49
Resoluble in 28% ammonia/methanol (4 : 1) for 4 hr	10	0	90	0	0	88	0	12
Resoluble in methanol for 20 hr	100	0	0	0	0	0	0	100
Resoluble in 1% formic acid/acetonitrile (1 : 4) for 20 hr	100	0	0	0	0	0	0	100

SR- and *RS*-ATDF did not change in any condition.

mers.[5] Regarding the stereoisomers of thalidomide, its teratogenic activity might be caused by its *S*-enantiomer. From these perspectives, in cases of unapproved pharmaceuticals containing stereoisomers such as ATDF, there is a safety concern that severe health damage might result from unpredicted activities of such stereoisomers in addition to these compounds' main activities. Therefore, in terms of health risk management, detection of a diastereomer of ATDF in this health food suggests the necessities of establishing an identification method for individual stereoisomers of other unapproved pharmaceuticals and of using that method to collect information related to their stereochemical profiles, such as optical purity. Furthermore, when health damage occurs because of the consumption of these unapproved pharmaceuticals, identification of the optical purity of the drugs must be performed to investigate the actual cause of such damage. Results of such identification would be useful to support medical treatment.

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